The Use of Collagen IV Immunohistochemistry In The Diagnosis of Bullous Pemphigoid

by

DR. ROXANNE CARON DE SILVA
DSLROX001

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Supervisors: Prof. Nonhlanhla P. Khumalo
Head of Department of Dermatology
University of Cape Town

Dr. Mzudumile R. Ngwanya
Consultant, Department of Dermatology
University of Cape Town

DEDICATION

For my husband, Kaveto and our son, Anthony

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DECLARATION

I, Roxanne Caron de Silva hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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THESIS ABSTRACT

Background

Autoimmune bullous dermatoses present with overlapping clinical features that require histopathological correlation. Immunofluorescence is the most routinely used reliable investigation for diagnosis but requires specialised equipment and is technically sophisticated. Collagen IV immunohistochemistry is reported as a reliable test for the diagnosis of epidermolysis bullosa acquisita whereby It stains the roof of a subepidermal blister and would be expected on the floor in bullous pemphigoid. This technique could be performed as an easily accessible alternative to direct immunofluorescence and has been used anecdotally at our hospital.

Aim

To investigate whether collagen IV immunohistochemistry can be used as a reliable histopathological confirmation of bullous pemphigoid.

Methods

Two major investigations:

1. A systematic literature search was undertaken of all studies describing the use of collagen IV immunohistochemistry and those comparing it with immunofluorescence in the diagnosis of bullous pemphigoid.
2. A retrospective study of patients diagnosed with bullous pemphigoid over 12 years seen at Groote Schuur Hospital was performed. Patient records that had results for both direct immunofluorescence and

collagen IV immunohistochemistry were selected. The positive percentage agreement was calculated.

Results

1. Two studies were found that investigated the use of collagen IV immunohistochemistry in bullous pemphigoid. All reported 33 (100%) cases demonstrated collagen IV at the floor of a subepidermal blister. Of these, 25/25 cases were in agreement with direct immunofluorescence and 7/8 with indirect immunofluorescence which were used as reference standard investigations.
2. In this study, collagen IV was positive in 96% (79/82) of cases and direct immunofluorescence was positive in 85% (72/82) of cases. A positive percentage agreement of 80.5% suggested a strongly positive test accordance.

Limitations

1. The literature search was limited to articles written in english only.
2. The retrospective design and the lack of controls without bullous pemphigoid made it impossible to calculate sensitivity and specificity as well as the kappa statistic.

Conclusion

Collagen IV immunohistochemistry is a valid, simple and widely available test which demonstrates accordance with routinely used direct immunofluorescence in the confirmation of bullous pemphigoid. Through clinical and histomorphological correlation, it may be a useful test in

resource-limited settings without facilities for direct immunofluorescence. However, larger controlled studies are warranted to confirm this.

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ABBREVIATIONS

BP230:	bullous pemphigoid antigen 1
BP180:	bullous pemphigoid antigen 2
IgE:	immunoglobulin E
H&E:	haematoxylin and eosin
IgG:	immunoglobulin G
C3:	complement 3
HIV:	human immunodeficiency virus
ELISA:	enzyme-linked immunosorbent assay
NBT/BCIP:	nitro blue tetrazolium/ 5-bromo-4-chloro-3-indolyl-phosphate
EBA:	epidermolysis bullosa acquisita
IF:	immunofluorescence
IgM:	immunoglobulin M

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. INTRODUCTION

1.1 Background

Autoimmune cutaneous blistering disorders may present with overlapping clinical features that require histopathological correlation to confirm the diagnosis. Direct immunofluorescence is used routinely in this regard but requires specialised equipment and is technically sophisticated, thus it is not widely available particularly in poor countries. Therefore, there is a need for other more easily accessible and accurate diagnostic tests for such disorders including bullous pemphigoid. In epidermolysis bullosa acquisita (EBA), a clinical differential diagnosis of bullous pemphigoid, collagen IV immunohistochemistry has already been used as a reliable and readily available confirmatory test by staining the roof of a subepidermal blister. Similarly, this technique may be used in the diagnosis of bullous pemphigoid by staining the floor of a subepidermal blister. Hence immunohistochemistry may offer an alternative to immunofluorescence in bullous pemphigoid. There is sparse literature in this regard thus the aim of this study is to investigate whether collagen IV immunostaining can be used as a reliable histopathological confirmation of clinical bullous pemphigoid.

1.2 Bullous pemphigoid

1.2.1 Definition and pathogenesis

Bullous pemphigoid is an acquired autoimmune blistering disorder of the skin characterised by large, tense cutaneous bullae. Attachment of autoantibodies against hemidesmosomal antigens namely BP230 (bullous pemphigoid antigen 1) and BP180 (bullous pemphigoid antigen 2 or collagen XVII) at the dermo-epidermal junction cause formation of subepidermal bullae, which can

be identified on histology of the skin.(1-3) Binding of the autoantibodies to these target-antigens results in complement activation, which in turn induces mast cell degranulation. It is also thought that immunoglobulin E (IgE) BP180 antibodies are bound to eosinophils and mast cells in lesional tissue of bullous pemphigoid. Exposure of the NC16A region of BP180 to these cells then causes mast cell degranulation thus releasing chemical mediators that recruit neutrophils and eosinophils to target tissue. Finally, proteolytic enzymes which breakdown dermal-epidermal cohesion and contribute to blister formation, are released by neutrophils and eosinophils which accumulate at the basement membrane.(4)

Neurological disease such as cerebrovascular disease, dementia, Parkinson's disease, epilepsy and multiple sclerosis have been reported as risk factors for developing bullous pemphigoid thus indirectly contributing to morbidity and mortality.(2) Bullous pemphigoid antigens or their isoforms have been detected in brain and neuronal tissue and it has been postulated that cross-reactivity of autoantibodies with brain and cutaneous antigens as well as exposure of hidden antigens in the brain cause an immune response. However, recent studies have not been able to confirm this mechanism.(5, 6) Possible triggering factors of bullous pemphigoid in those with neurologic disease include drug intake, pressure sores, traumatic events and immunity aging.(6)

In a minority of bullous pemphigoid cases, other recognisable inducing factors can be identified. Various drugs (particularly those with sulfhydryl groups), physical agents, viral infections and diet have been associated with bullous pemphigoid in genetically predisposed individuals.(4)

1.2.2 Clinical Presentation

Bullous pemphigoid occurs equally in both sexes and most commonly affects the elderly but it also presents in the young, particularly children.(2, 7) Tense

cutaneous bullae overlying erythematous or normal-appearing skin that heal without scaring are typical of bullous pemphigoid (Figures 1-2). Blisters may be localised or generalised with a tendency to involve flexural surfaces and may contain clear or blood-stained fluid. This may be preceded by weeks to months with erythema and/or urticarial plaques which may or may not be pruritic. The oral mucosa, more frequently than genital mucosa, may also be involved with small tense blisters or erosions in up to 50% of cases.(1-3, 7) Rare atypical presentations of bullous pemphigoid have been described including annular erythema-like, erythema multiforme-like, pemphigoid nodularis, lichen planus pemphigoides, vesicular, erythrodermic, dyshidrosiform and vegetans bullous pemphigoid. Localised variants include pretibial, vulvar and umbilical bullous pemphigoid. (8)

1.2.3. Differential Diagnoses

There are several other blistering disorders of the skin with differing aetiologies, pathogeneses and prognoses which may sometimes mimic bullous pemphigoid.(9) These include autoimmune blistering disorders such as linear IgA bullous dermatosis, cicatricial pemphigoid, pemphigoid gestationis and bullous lupus erythematosus. Genetic bullous disorders such as the epidermolysis bullosa group and other conditions such as insect bite allergy, burns, erythema multiforme, contact dermatitis and certain viral and bacterial infections may also present with blistering.(2) In practice, patients do not always present with classical morphology and distributions. This may be due to the severity and stage of disease at the time of examination as well as the effect of previous treatments used, further complicating the clinical picture.(10-12) The management of each condition will differ; therefore an accurate diagnosis is essential.

1.2.4 Investigations in bullous pemphigoid

Histopathology (routine):

The diagnosis of bullous pemphigoid is often suspected clinically but should be correlated histopathologically.(3, 7, 10, 11, 13, 14) Subepidermal clefting with a mixed dermal inflammatory infiltrate predominantly containing eosinophils and occasionally neutrophils are demonstrated on histology of a fresh blister that is formalin fixed, paraffin embedded before sectioning and stained with haematoxylin and eosin (H&E) (Figures 3-4). However, the most widely used reliable confirmation is achieved with immunopathology by direct or indirect immunofluorescence.(2, 3, 7, 12, 15)

Immunofluorescence:

Direct immunofluorescence is performed on perilesional skin where immune deposits are most readily detected and specimens are transported fresh, in normal saline or in Michel's transport medium to the laboratory where they are cryostat embedded for frozen sectioning.(2, 3, 7, 9, 12) This technique involves the conjugation of an antibody with a fluorochrome that is directed against a tissue antigen (manually or via automation) and detected using ultraviolet light microscopy.(12, 16) The site, strength and pattern of deposition of the immune complexes will accurately diagnose the relevant autoimmune bullous dermatosis. Linear deposits of immunoglobulin G (IgG) autoantibodies and or complement 3 (C3) will be detected at the dermo-epidermal junction in bullous pemphigoid (Figures 5-6).(2, 3, 7, 9, 12, 16)

The differential diagnosis for the deposition of IgG and or C3 at the basement membrane zone in a subepidermal blister other than bullous pemphigoid includes cicatricial pemphigoid, pemphigoid gestationis, epidermolysis bullosa acquisita and bullous lupus erythematosus. Hence, in order to differentiate bullous pemphigoid from other autoimmune bullous dermatoses, the salt split-skin technique combined with immunofluorescence can be used.

This technique has the advantage of improving the sensitivity of direct immunofluorescence.(2, 3, 7, 11, 13, 15) Skin is incubated in one molar sodium chloride solution resulting in separation of the epidermis from the dermis at the level of the lamina lucida at the basement membrane zone. In this way, immunofluorescent deposits of antibodies become visible on the epidermal side of the blister as seen in bullous pemphigoid in more than 90% of patients (2, 3, 7, 11, 13, 15, 17) and on the dermal side of the blister in EBA, bullous systemic lupus erythematosus and cicatricial pemphigoid.

Direct immunofluorescence specimens can be processed manually if automation is unavailable but costly equipment such as a cryostat machine for frozen sectioning, deep freezers at -80°C to store these sections until staining and an ultraviolet light attachment for light microscopy is required. Two biopsies are necessary to perform routine H&E staining as well as direct immunofluorescence because the later cannot be performed on paraffin embedded tissue. Immunofluorescence does not permitt the examination of cytological detail as well as histomorphology and results are often equivocal due to poor resolution.(12, 16, 18-20) Furthermore, fluorescence decays with time and with exposure to light therefore slides cannot be reviewed at a later stage.(12, 20) Processing of specimens from known human immunodeficiency virus (HIV)-reactive patients is avoided in some laboratories due to the risk of accidental transmission of HIV and hepatitis B whilst handling fresh infectious tissues under sharp sectioning blades. In developing countries with limited resources, some of which have the highest burdens of HIV and hepatitis B, this may be impractical thus further emphasising the need for the development of other easily accessabile confirmatory techniques.

Indirect immunofluorescence of blister fluid, serum or urine is another immunopathological technique used to confirm and monitor bullous pemphigoid. Circulating IgG or C3 bind in a linear pattern at the basement membrane zone of squamous epithelia substrates such as normal skin or

monkey oesophagus (Figure 7).(2, 3, 7, 11) Although this test is sensitive and highly specific (21), it is only performed in a few centres globally.

Other less commonly used immune-based tests:

Additional specialised diagnostic techniques for bullous pemphigoid, used predominantly for research purposes, include the enzyme-linked immunosorbent assay (ELISA) for serum levels of antibodies to BP180 and BP230. The NC16A domain, a pathogenic epitope of the BP180 antigen, is also used to correlate disease activity with antibody titres for selected cases only.(2) Immunoblot and immunoprecipitation are also highly specific research tools used to demonstrate the reactivity of IgG from patient sera with 180kDa and 230kDa bullous pemphigoid antigens (BP180 and BP230 respectively).(3, 22)

Lastly, laser scanning confocal microscopy allows precise localisation of in vivo-bound IgG and has been used as an extremely specialised, rapid method for differentiating bullous pemphigoid from EBA and cicatricial pemphigoid.(23) In vivo-bound IgG is localised on the epidermal side of laminin 5 and co-localised with B4 integrin in bullous pemphigoid, whereas in EBA, the IgG is present on the dermal side of collagen IV. In cicatricial pemphigoid, IgG is bound between laminin 5 and type IV collagen.(23)

A prospective study undertaken by Chan et al compared these tests in 23 cases of bullous pemphigoid diagnosed clinically. They found 91% (21/23) demonstrated linear deposits of IgG and C3 along the dermo-epidermal junction on direct immunofluorescence whereas 96% (22/23) were positive on serum indirect immunofluorescence, all of which bound to the epidermal side of the cleft on salt-split skin. The BP180 NC16A antibody was also detected in 96% (22/23) using the ELISA technique but the immunoblot was superior with positive reactivity to BP 180 and/or BP230 in 100% (23/23). However, it was described as being technically very demanding.(24)

1.3 Immunohistochemistry

1.3.1 Techniques in Immunohistochemistry

Immunohistochemistry is a histopathological technique, not routinely performed in bullous pemphigoid, whereby specific antibodies are used to localise desired antigens within fresh or frozen tissue specimens.(19) Significant advances in the use of immunohistochemistry were made in the early 1990's and in 1991 a breakthrough made it possible to perform antigen retrieval in formalin-fixed, paraffin-embedded tissues as well.(25) This sparked new insights and interest in the field and broadened the test's capabilities.(25) An increase of publications in this regard was prompted; including that regarding the use of collagen IV immunohistochemistry. The technique may be performed manually (using precise laboratory protocols) or in automated platforms that have the advantage of enhanced quality and reproducibility.(16) Monoclonal or polyclonal antibodies can be used but differ in their binding affinities and specificities such that monoclonal antibodies, which react with only one epitope, are highly specific but less sensitive versus polyclonal antibodies that react with multiple epitopes of a single antigen becoming less specific but highly sensitive.(19, 26)

The initial and traditional direct immunohistochemistry technique involves the reaction of labeled antibodies directly with tissue antigen. Indirect techniques include the two-step technique whereby a labeled secondary antibody is directed against an unlabeled primary antibody that is bound to the relevant antigen. The three-step indirect technique, also called the labeled streptavidin-biotin method, is another indirect method involving the attachment of an unconjugated primary antibody to the tissue antigen creating an antigen-antibody complex. Secondly, a biotinylated secondary antibody is directed against the primary antibody followed lastly by enzyme-labeled streptavidin or a complex of enzyme-labeled biotin and streptavidin

that is bound to the secondary antibody. The enzyme used may be horseradish peroxidase or alkaline phosphatase. In addition, the relevant chromogens are used for detection, such as diaminobenzidine or 3-amino-9 ethylcarbazole used in the peroxidase method or indole reagents, naphthol fast red or nitro blue tetrazolium/ 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) chromogens used in the alkaline phosphatase-streptavidin method.(16, 27)

Although the streptavidin-biotin method has been used widely, the presence of endogenous biotin within specimens may result in unwanted background staining. Other newer and improved polymer detection systems that are quicker, reliable and reproducible with greater sensitivity have been developed including the Enhanced Polymer One Step technique. This popular method involves the attachment of multiple enzyme molecules and specific primary antibodies to a dextran backbone processed via manual or automated immunohistochemistry.(16, 27)

False positive results within immunohistochemistry may be related to 1) chromogen entrapment due to inadequate rinsing, prolonged chromogen time as well as chatter, tears, folds, wrinkles and poor adhesion of sections to slides; 2) precipitation of chromogen which may be prevented by adequate filtering and 3) contaminants acquired during handling water baths, tissue sections and slides with ungloved hands. In contrast, false negative results can occur due to tissue processing errors such as 1) incomplete paraffinisation resulting in suboptimal staining because of incomplete tissue penetration by antibodies; 2) over-digestion of tissue sections by proteolytic enzymes that destroy tissue antigens; 3) incorrect temperature of reagents; 4) expired antibodies; 5) inappropriate dilutions and 6) suboptimal storage of antibodies. Nonetheless, errors such as these may be easily avoided by adhering to precise laboratory protocols when performing immunohistochemistry manually as well as on automated platforms.(16)

1.3.2 Collagen IV Immunohistochemistry and autoimmune bullous disorders

Collagen IV immunohistochemistry may be performed in the diagnosis of bullous pemphigoid. Autoantibodies target hemidesmosomal antigens forming a cleft above the lamina densa where collagen IV, part of the normal structure of the basement membrane zone, is located. In this way, immunostaining of collagen IV should be detected on the floor of subepidermal blisters in bullous pemphigoid (Figure 8). This is supported by Pardo *et al* who reported that immunohistochemistry is a reliable, fast and readily available laboratory tool to determine the level of collagen IV within a subepidermal blister.(18)

The usefulness of collagen IV immunohistochemistry has been described in dogs to confirm the diagnosis of EBA but it is also used in humans.(18, 28) EBA is an autoimmune blistering condition characterized by autoantibodies against collagen VII in the sublamina densa of the dermo-epidermal junction (below the lamina densa where collagen IV is located) resulting in a cleft at this level. Therefore, collagen IV immunostaining will be detected on the roof of subepidermal blisters formed in EBA.

Collagen IV immunohistochemistry is a rapid simple tool that utilises readily available reagents and can be performed locally in most pathology laboratories.(18) Apart from bullous pemphigoid, porphyria cutanea tarda, dermatitis herpetiformis and adult linear IgA bullous dermatosis may also present with subepidermal blisters which demonstrate collagen IV immunostaining at the floor.(18, 29, 30) However, other defining histological features such as cell-poor blisters, festooning and hyalinised vessels in porphyria cutanea tarda can differentiate these subepidermal blisters from those of bullous pemphigoid. In addition, investigations such as urinary porphyrins are diagnostic in porphyria and cutaneous features such as

scarring and photosensitivity are rarely confused with bullous pemphigoid. Patients with dermatitis herpetiformis may also have features of gluten-intolerance that will distinguish it from other autoimmune bullous dermatoses and linear IgA demonstrates predominantly neutrophil rich blisters on histology.

2. SYSTEMATIC LITERATURE REVEIW

2.1 Objective

To identify and review studies that have investigated the use of collagen IV immunohistochemistry alone or compared with immunofluorescence in the diagnosis of bullous pemphigoid.

2.2 Literature search strategy

Searches were conducted in five databases to identify recent publications until December 2016: Medline via PubMed, Scopus, Academic Search Premier, Africa-Wide Information via EBSCO host and Google Scholar. The following search terms were used in various combinations: bullous dermatoses, vesicobullous skin diseases, bullous pemphigoid, immunohistochemistry and collagen IV (Table 1). Searches were limited to English articles concerning humans within the field of medicine and no filter was set regarding publication-date. Abstracts of identified documents were read and the full text of relevant documents were retrieved for inclusion in the review. Reference lists of retrieved documents were also searched to identify additional publications. All articles regarding the use of collagen IV immunohistochemistry in patients with bullous pemphigoid were included. A summary of the database searches is set out below.

2.3 Results of literature search

Two diagnostic studies were found that investigated the use of collagen IV immnohistochemistry in a total of 33 participants with bullous pemphigoid. All cases stained the floor of a subepidermal blister (Table 2). Of these, 25/25 cases were in agreement with direct immunofluorecence and 7/8 cases with indirect immunofluorescence, which were used as reference standard investigtions.

In the first study, Pardo *et al* performed a prospective study of 25 cases of bullous pemphigoid, 12 cases of porphyria cutanea tarda and three cases of dermatitis herpetiformis, which were all confirmed on direct immunofluorescence. Five clinically established longstanding cases of EBA that had been confirmed by unspecified investigations at the time of diagnosis were also included. Sections of all cases were stained with H&E to confirm the presence of subepidermal blisters. Thereafter, the indirect avidin-biotin-peroxidase immunohistochemical technique was performed using polyclonal rabbit antibodies against collagen IV (primary antibody) and goat anti-rabbit IgG (secondary antibody) on deparaffinised tissue sections that had been formaldehyde-fixed and paraffin-embedded prior to processing.(18) Positive collagen IV immunostaining was demonstrated on the floor of 100% of the bullous pemphigoid cases (25/25), the porphyria cutanea tarda cases (12/12) and the dermatitis herpetiformis cases (3/3). In all of the EBA cases (5/5), collagen IV stained the roof of the subepidermal blisters.(18) This small study suggests that collagen IV immunostaining is not only consistent with direct immunofluorescence but reliably distinguishes between autoimmune blistering disorders based on target antigen location on either the floor or roof of the blister.

The second study, undertaken by Bowszyc-Dmochowska *et al* prospectively, tested eight cases of bullous pemphigoid. Two cases of EBA and five controls (including two cases of dermatitis herpetiformis, two cases of bullous

lichen sclerosis and one case of localised recessive dystrophic epidermolysis bullosa inversa) were also included. All cases were confirmed with salt-split skin indirect immunofluorescence on human skin and immunoblot. Tissues were formalin-fixed and paraffin-embedded before processing and routine H&E staining was performed on each section. The avidin-biotin immunohistochemical technique was also performed but pre-diluted mouse monoclonal antibodies and anti-mouse biotinylated antibodies were utilised. In all cases of bullous pemphigoid (8/8), collagen IV stained positively on the floor of the subepidermal blisters. All cases were also positive on the immunoblot but the salt split skin indirect immunoflourence demonstrated fluorescence on the epidermal side of the split in 86% (7/8) and on both the epidermal and dermal side of the split in one case (1/8). In the EBA cases, collagen IV stained the roof of the blister clearly with lighter staining on the floor in one and the other stained equivocally. Two of the control cases, namely dermatitis herpetiformis, stained at the base of the blisters whereas the other control cases stained positively on the roof.(29)

3. DISCUSSION

The results of both studies confirm that the location of collagen IV at the basement membrane zone in relation to a subepidermal blister can be identified in routinely processed tissue i.e. formaldehyde-fixed and paraffin-embedded. They are also in keeping with the theoretical background of subepidermal blister formation in bullous pemphigoid. Overall, collagen IV demonstrates good accordance with the reference standards thus highlighting the potential role of collagen IV immunohistochemistry as a sensitive test in the confirmation of clinically suspicious bullous pemphigoid.

Test Accuracy:

Immunofluorescence (on salt-split skin) and immunoblot, both highly reliable techniques in the diagnosis of autoimmune bullous dermatoses (2, 3, 7, 12,

15, 22), were utilised as reference standards. The collagen IV results of all cases of bullous pemphigoid, porphyria cutanea tarda, dermatitis herpetiformis, bullous lichen sclerosis and localised recessive dystrophic epidermolysis bullosa inversa demonstrated positive agreement with these tests thus verifying collagen IV as an accurate test. One exception was a case of bullous pemphigoid, where IgG antibodies bound to both epidermal and dermal sides of the split on indirect immunofluorescence, which is out of keeping with the theoretical background. However, the same case was confirmed on immunoblot thus the unexpected result might represent the presence of the rarely described BP200 dermal-binding antigen (31) or an artifact due to tissue handling and processing errors.

Test Validity:

In the first study, non-immune rabbit serum was used in antibody control sections and collagen IV detected around blood vessels and adnexae served as an internal control. The second study did not report on antibody and internal controls but utilised clinical control cases. Although not specified, the additional cases in the first study also served as clinical controls against bullous pemphigoid. The results of all positive controls in both studies were in keeping with the theoretical expectations of subepidermal blister formation in each disorder and they were in agreement with the reference standards used thus helping to validate collagen IV.

Bias:

The number of investigators and whether or not they were blinded was not reported in either study. This is important as the results of immunofluorescence and immunohistochemistry techniques are interpreter dependent and will influence observer bias. In this way, the accuracy of the index test, collagen IV, may have been overestimated. However, in the first study, serial dilutions of the antiserum were performed to determine optimal antibody concentrations and hydrogen peroxidase was utilised to reduce background staining that commonly occurs in the avidin-biotin method, as

previously described. The second study utilised pre-diluted commercial antibodies and sections were treated with alpha-chymotrypsin to enhance the reactivity of collagen IV at the basement membrane zone. These techniques might have helped to reduce the chance of observer bias by ensuring adequate staining quality that would make interpretation of the immunostaining easier.

Sensitivity and Specificity:

Both studies, although small, included patients with different autoimmune bullous dermatoses. No false positive or false negative collagen IV results were detected in either study in the bullous pemphigoid group. This is in line with good test sensitivity and specificity but larger sample sizes including disease free controls are required to confirm this observation.

Positive test agreement of all cases of bullous pemphigoid with the reference standards suggests that collagen IV on the floor of a subepidermal blister is a sensitive test. However, because more than one condition may demonstrate collagen IV on the floor, clinical-pathologic correlation is still crucial in order to confirm the results. The findings of both studies also suggest that collagen IV staining the roof of a subepidermal blister may be used as a specific test to exclude bullous pemphigoid, as none of these cases presented with collagen IV on the roof.

Research gaps:

Both studies reported similar results in the bullous pemphigoid groups, but only include a total of 33 patients. Prospective studies are required to better regulate sample collection and processing in order to avoid artifacts that may influence test results. Furthermore, studies with more than one blinded investigator would provide more accurate results by avoiding observer bias. Ideally, larger sample sizes of confirmed cases of bullous pemphigoid and disease-free controls are required to verify test reliability and reproducibility

as well as to calculate test sensitivity and specificity. In addition to this, positive and negative predictive values might also be calculated. These values will provide a measure of the proportion of people with a positive test who have the condition, and vice versa, in relation to test accuracy and the prevalence of bullous pemphigoid.

4. CONCLUSION AND RECOMMENDATIONS

We found two published studies (reporting only 33 patients) comparing collagen IV immunohistochemistry and immunofluorescence in the diagnosis of bullous pemphigoid. In spite of promising results from these two small studies from 1990 and 1997, no other work has been published on the subject. In our clinical practice collagen IV is often used in addition to direct immunofluorescence in the diagnosis of bullous pemphigoid. The current study (Chapter 2 of this thesis), entitled “The use of collagen IV immunohistochemistry in the diagnosis of bullous pemphigoid”, includes a larger than published patient number and it will be the first to our knowledge to investigate collagen IV test agreement with direct immunofluorescence in the diagnosis of bullous pemphigoid at a hospital in Africa and the rest of the world since the late 1990’s. The results will provide insight into the value of collagen IV immunohistochemistry, which is more readily available than direct immunofluorescence, in the diagnosis of bullous pemphigoid.

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6. TABLES

Table 1

Literature Search Stratergy

Database searched	Search Terms	Limits	Results	Used
Medline/PubMed	(immunohistochemical method) and (bullous dermatoses) MeSH terms (methods, skin, diseases, vesicobullous, vesicobullous skin diseases)	English	112	1
Medline/PubMed	(immunohistochemical method) and (bullous pemphigoid) MeSH terms (Methods)	English	29	2
Medline/PubMed	(collagen IV) and (bullous pemphigoid)	English	141	1
Scopus	(immunohistochemical method) and (bullous dermatoses)	English	5	0
Scopus	(immunohistochemical method) and (bullous pemphigoid)	English	37	2
Scopus	(collagen IV) and (bullous pemphigoid)	English, Humans, Medicine,	146	2
Academic Search Premier	(immunohistochem*) and (bullous*)	English	163	1
Academic Search Premier	(immunohistochem*) and (bullous pemphigoid)	English	91	1
Africa-Wide Information/ EBSCO host	(immunohistochem*) and (bullous*)	English	5	0
Africa-Wide Information/ EBSCO host	(immunohistochem*) and (bullous pemphigoid)	English	0	0

Table 2

Literature Search Results

Author, Year & Country	Investigation & Participant Number	Outcome
Pardo *et al*	Collagen IV	**Positive on floor of subepidermal blister in:**
1990	25 cases	25/25 cases of bullous pemphigoid,
USA(18)		12/12 cases of porphyria cutanea tarda,
		3/3 cases of dermatitis herpetiformis
		Positive on roof of subepidermal blister in:
		5/5 cases of EBA
	Direct immunofluorescence	**Positive in 25/25 cases**
	25 cases	
Bowszyc-Dmochowska	Collagen IV	**Positive on floor of subepidermal blister in:**
1997	8 cases	8/8 cases of bullous pemphigoid and
Poland(29)		1/3 cases of EBA
		Positive on roof of subepidermal blister in:
		1/3 cases of EBA and
		Equivocal in 1/3 cases of EBA
	Direct immunofluorescence	**Positive in 7/8 cases**
	8 cases	

7. FIGURES

Figure 1: Tense cutaneous blisters and erosions with haemorrhagic crusts on an erythematous base in a patient with bullous pemphigoid

Figure 2: Tense cutaneous blisters overlying urticarial plaques in a patient with bullous pemphigoid

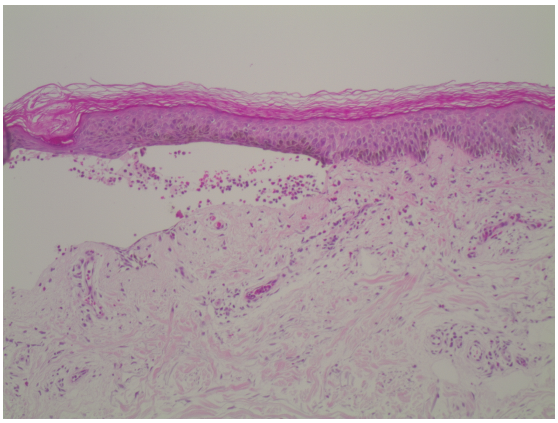

Figure 3: Haematoxylin & eosin 100x objective magnification: The section shows a cell rich subepidermal blister containing inflammatory cells

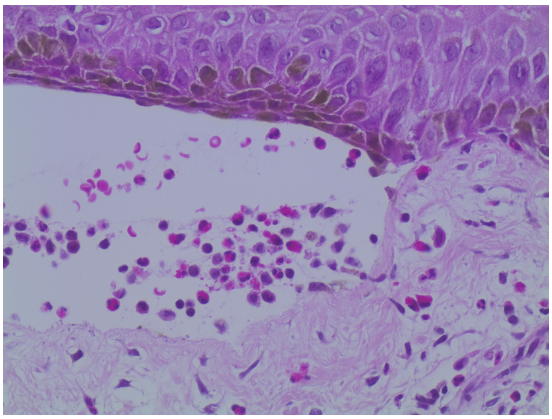

Figure 4: Haematoxylin & eosin 400x objective magnification: The section shows a cell rich subepidermal blister containing numerous eosinophils

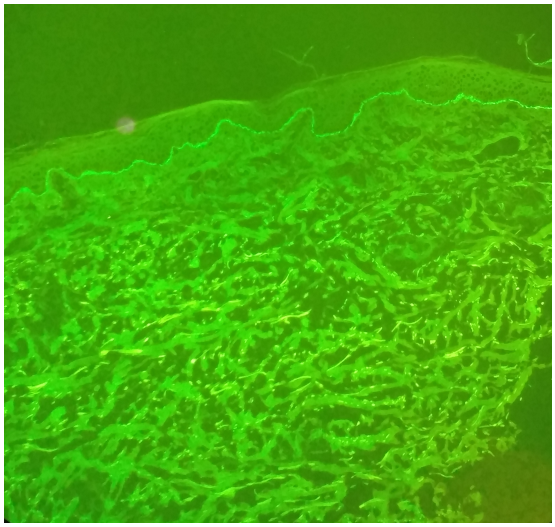

Figure 5: 100x objective magnification of IgG direct immunofluorescence demonstrating 3+ linear staining at the dermo-epidermal junction

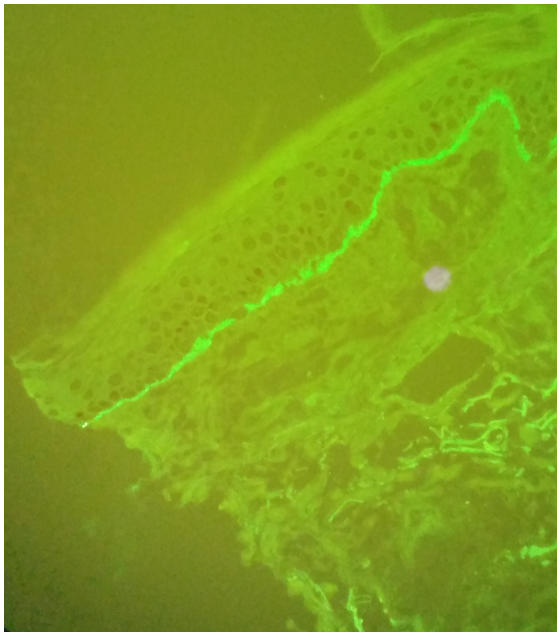

Figure 6: 100x objective magnification: C3 direct immunofluorescence demonstrating 3+ linear staining at the dermo-epidermal junction

(32)

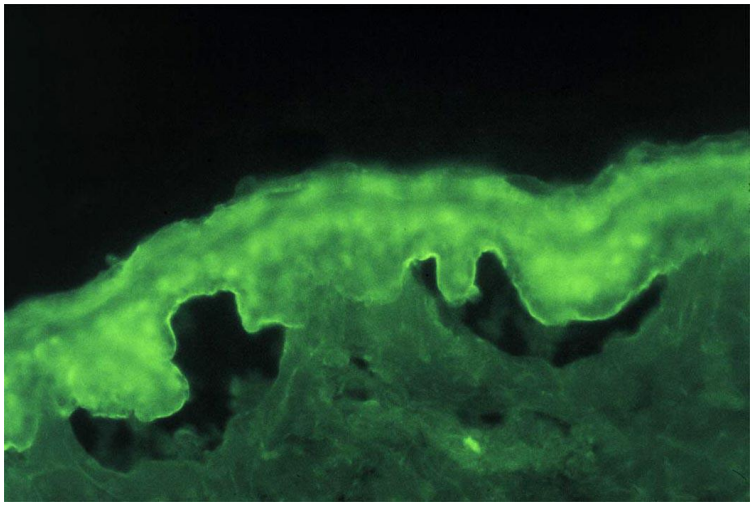

Figure 7: Indirect immunofluorescence performed on salt-split normal human skin substrate with the serum from a patient with bullous pemphigoid demonstrating circulating IgG autoantibodies binding to the epidermal side of the dermo-epidermal junction

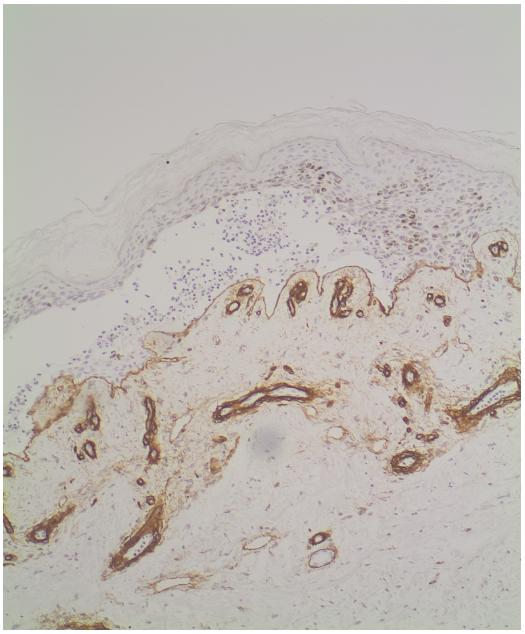

Figure 8: 100x objective magnification: Collagen IV immunohistochemistry stain showing positive staining at the floor of the blister cavity. An overlying subepidermal blister is noted.

CHAPTER 2

PUBLICATION-READY MANUSCRIPT FOR SUBMISSION TO THE BRITISH JOURNAL OF DERMATOLOGY

The Use of Collagen IV Immunohistochemistry In The Diagnosis of Bullous Pemphigoid

R.C. de Silva[1], M.R. Ngwanya[1], R. Roberts[2], N.P. Khumalo[1]

[1] Division of Dermatology, Department of Medicine, University of Cape Town, , South Africa
[2] Division of Anatomical Pathology, National Health Laboratory Service and University of Cape Town, South Africa

Word count: 1197
Table count: 1
Figure count: 3

Correspondance:

R.C. de Silva

Address:
Division of dermatology, G23
Groote Schuur Hopsital
Main Road
Observatory
7935
Cape Town
South Africa

Tel: +27 81 5861910
Email: rcdesilva@yahoo.co.uk

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Conflicts of interest: None to declare

Statements (70 words):

- What is known about topic?

Immunofluorescence is the most relied on investigation in the diagnosis of bullous pemphigoid but it requires specialised equipment thus limiting its use in resource-limited settings.

- What does this study add?

This study demonstrates the potential use of collagen IV immunohistochemistry as a simple, reliable and accessible alternative to direct immunofluorescence in the diagnosis of bullous pemphigoid.

Abstract

Autoimmune bullous dermatoses present with overlapping clinical features that require histopathological correlation. Immunofluorescence is the most reliable but requires expensive equipment and expertise. Collagen IV immunohistochemistry, which is performed on formalin fixed tissue, has been compared to immunofluorescence in the diagnosis of bullous dermatoses. A systematic search of five databases identified two small studies comprising 33 participants which showed good agreement between immunofluorescence and collagen IV in bullous pemphigoid. No other study since 1997 has confirmed of refuted these findings.

Methods

A retrospective study of all patients diagnosed with bullous pemphigoid over 12 years within a tertiary dermatology department was performed. Patient records with both collagen IV and direct immunofluorescence results were selected and the positive percentage agreement was calculated.

Results

Collagen IV was positive in 96% (79/82) and direct immunofluorescence in 85% (72/82) of patients with clinical bullous pemphigoid. A positive percentage agreement of 80.5% suggests a strong test accordance.

Limitations

The retrospective design and lack of controls without bullous pemphigoid made it impossible to calculate sensitivity and specificity or to confirm test agreement with the kappa statistic.

Conclusion

This is the largest study, comparing direct immunofluorescence and collagen IV in the same patients diagnosed with bullous pemphigoid. Collagen IV immunohistochemistry can be performed on one biopsy also used for routine histology. It is a simple, widely available tool that has accordance with and may be more sensitive than direct immunofluorescence in the diagnosis of bullous pemphigoid. Confirmation in larger controlled studies is essential as collagen IV immunohistochemistry is accessible in resource-limited countries.

Introduction

Bullous pemphigoid is the most common acquired autoimmune blistering dermatosis of the skin in Western Europe (1) and globally. It is characterised by large, tense cutaneous bullae which commonly affect the elderly but may present in children.(2) Cutaneous blistering diseases with differing aetiologies, pathogeneses and prognoses including other autoimmune and genetic bullous diseases may have clinical features which overlap with that of bullous pemphigoid. This might be due to the severity and stage of disease at the time of examination and the effect of previous treatments used.(3-5) Therefore, in order to confirm the diagnosis, histopathological correlation is required.(2) Routinely, immunofluorescence is considered most reliable, but requires specialised equipment and expertise. Thus it is not widely accessible particularly in poor countries.

A systematic literature search of five databases for all studies written in English that describe the use of collagen IV immunohistochemistry and those comparing it with immunofluorescence in the diagnosis of bullous pemphigoid identified only small two studies. All reported 33 (100%) bullous pemphigoid cases demonstrated collagen IV on the floor of a subepidermal blister. Of these, 25/25 cases concurred with direct immunofluorescence (6) and 7/8 with indirect immunofluorescence (7) which were used as reference standard investigations. It is noteworthy that in spite of good agreement between immunofluorescence and collagen IV immunohistochemistry, no data confirming or refuting this finding in larger studies has been published since 1997. In our clinical practice collagen IV is often used in addition to direct immunofluorescence in the diagnosis of bullous pemphigoid.

Methods

A retrospective record analysis was performed of all inpatients and outpatients with a clinical diagnosis of bullous pemphigoid between February 2003 and May 2015 within a dermatology department at a specialist referral hospital in South Africa.

Participants with the following were included: 1) Tense cutaneous blisters with or without an erythematous base and with or without pruritus; 2) a subepidermal blister with associated eosinophils on histology; 3) collagen IV immunohistochemistry staining from biopsy of a blister and 4) direct immunofluorescence performed on perilesional skin.

Commercially available Ventana polyclonal mouse and polyclonal goat primary antibodies were used in the collagen IV immunohistochemistry and direct immunofluorescence techniques respectively. Both methods were routinely performed on the Ventana Benchmark and Benchmark Ultra automated platforms. Collagen IV staining the floor of a subepidermal blister was considered positive as demonstrated (fig. 1). Direct immunofluorescence of perilesional skin biopsies reported as demonstrating immunoglobulin G (IgG) (fig. 2) and/or complement 3 (C3) at the basement membrane zone fluorescing in a linear pattern was accepted as a positive test. The strength of fluorescence was graded 1+ to 3+, where 1+ is weak and 3+ is strong. Negative direct immunofluorescence was defined as no fluorescence, detection of IgA and/or IgM only at the basement membrane zone or equivocal findings. The positive percentage agreement between collagen IV immunohistochemistry and direct immunofluorescence was analysed.

Results

In total, 82 participants were included in the study; 77% (63/82) were female, 27% (22/82) were male. The mean age was 69 years old.

Collagen IV immunohistochemistry was positive in 96% (79/82) of cases (table 1) and direct immunofluorescence was positive in 84% (69/82) of which IgG and C3 stained positively in 64% (44/69), 9% (6/69) for IgG only and 15% (10/69) for C3 only (fig 3). A positive percentage agreement of 80.5% suggests a strongly positive test accordance.

Discussion

Our results reflect that collagen IV immunohistochemistry has good accordance with direct immunofluorescence in the diagnosis of bullous pemphigoid. Only three cases in our study did not detect collagen IV at the base of a subepidermal blister. No blister was present in all of these sections thus the test could not be interpreted. Therefore, with clinical-pathologic correlation, collagen IV performed on a blister is a useful alternative when direct immunofluorescence is unavailable.

Immunohistochemistry is a rapid and simple tool that utilises readily available reagents and can be performed locally in most pathology laboratories.(6) However, there have been surprisingly few studies regarding the use of collagen IV. We found two studies of 25 and eight confirmed cases of bullous pemphigoid respectively that demonstrated positive collagen IV staining the floor of all subepidermal blisters. All cases in the former study were in agreement with direct immunofluorescence and 7/8 cases in the later with indirect immunofluorescence.(6, 7) Both studies confirm the validity of this test and verify that collagen IV can be used to determine the level of a

subepidermal blister at the basement membrane zone on routinely formalin-fixed and paraffin-embedded tissues. Thus only one biopsy is required for routine histology and immunohistochemistry. Furthermore, good accordance with immunofluorescence and the lack of false positives in these studies is in keeping with good test sensitivity. The results also suggested that bullous pemphigoid can be excluded if collagen IV stains the roof of a subepidermal blister. Overall, these studies are limited by their retrospective nature, small sample sizes and observer bias that might have lead to overestimation of test accuracy, but our study findings are in support of their outcomes.

Direct immunofluorescence has long been considered the most reliable investigation in the diagnosis of bullous pemphigoid.(1, 5, 8-10) One study was found that investigated the use of direct immunofluorescence in 227 participants with bullous pemphigoid confirmed by any two investigations including histopathology, indirect immunofluorescence and enzyme-linked immunosorbent assay against 448 disease free-controls. They calculated a sensitivity and specificity of 90.8% (CI: 86.2%-94.2%) and 98% (CI: 96.2%-99.1%) respectively. This was a large study that was only limited by its retrospective nature.(11)

Despite the efficacy of direct immunofluorescence, contrary to immunohistochemistry, it is not as readily available and is more sophisticated. Specialised equipment such as a cryostat machine for frozen sectioning, deep freezers to store sections until staining and an ultraviolet light attachment for light microscopy are required. Two biopsies, one of a blister for formalin-fixation and paraffin-embedding to perform routine histology and another fresh perilesional biopsy for direct immunofluorescence are required. Skilled pathologists to perform and interpret this test are vital as the site, strength and pattern of fluorescence varies amongst several autoimmune bullous dermatoses. Furthermore, fluorescence decays with time and on exposure to light whereas immunohistochemistry is permanent.(5) Another disadvantage is the

increased risk of human immunodeficiency virus and hepatitis transmission, diseases which place a high burden on poor and developing countries, because of handling fresh tissues on sectioning blades in this technique.

Limitations

Due to the retrospective nature of this study, clinical information was dependent on accurate record keeping by doctors and sampling error as well as biopsy technique could not be accounted for. The sample size, which is larger than preceding studies, but still too small together with the lack of clinical controls, made it impossible to calculate sensitivity, specificity and the kappa statistic.

Conclusion and recommendations

Collagen IV immunohistochemistry is a simple, widely available test that has good accordance with direct immunofluorescence. Through clinical and histomorphological correlation, it may be a useful confirmatory tool in resource-limited settings where immunofluorecence is not possible. Furthermore, collagen IV is not only more accessible, but it can be performed on the same biopsy used for routine histology thus further reducing the cost of diagnostic confirmation. However, larger controlled studies are warranted.

Conflict of interest

None to declare

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Table

Table 1

	Collagen IV		
	Positive	Negative	Total
Direct Immunofluorescence			
Positive	66	3	69
Negative	13	0	13
Total	79	3	82

Figures

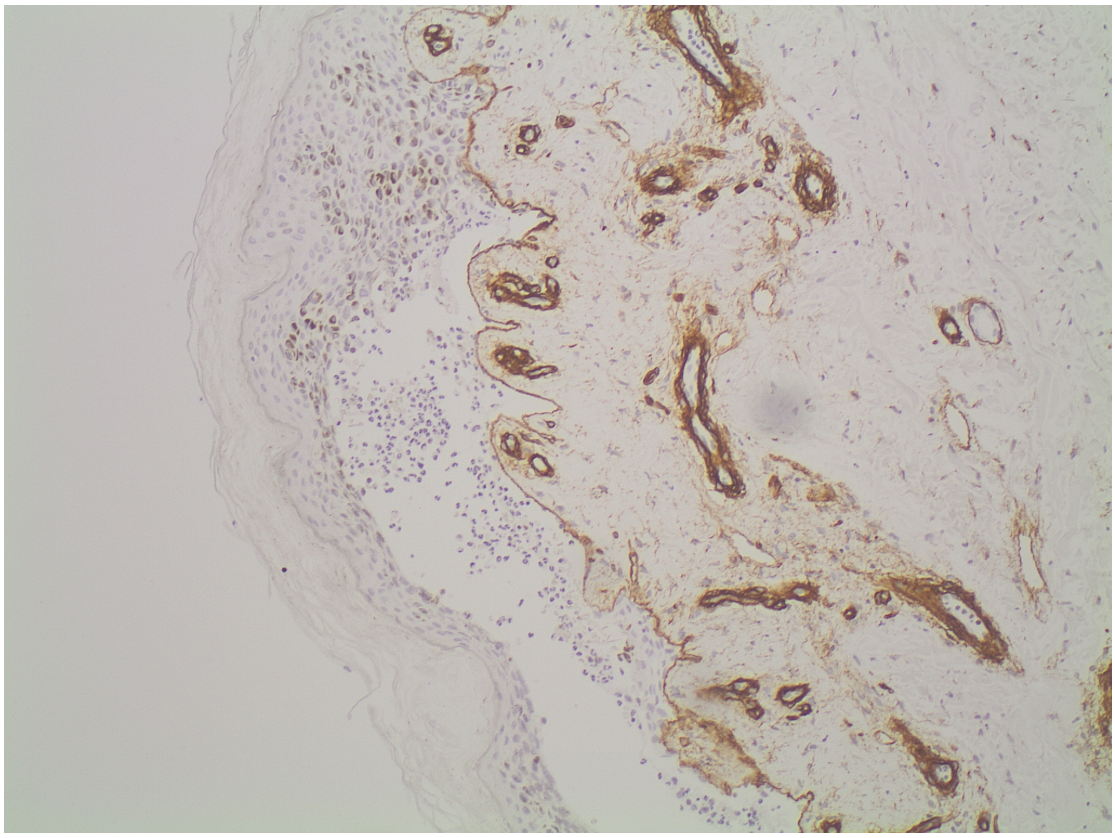

Figure 1: **100x objective magnification: Collagen IV immunohistochemistry stain showing positive staining at the base of the blister cavity. An overlying subepidermal blister is noted.**

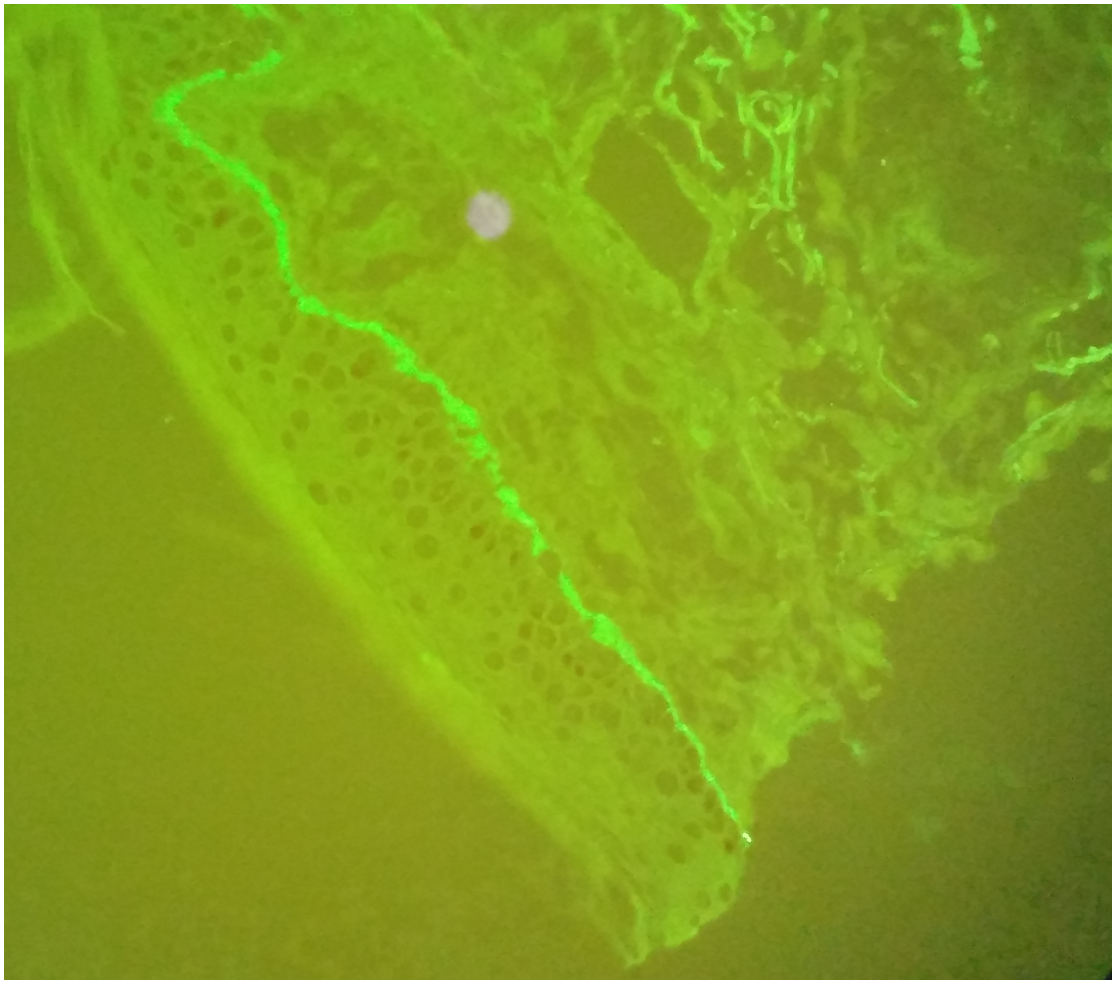

Figure 2: **100x objective magnification: C3 direct immunofluorescence demonstrating 3+ linear staining at the dermo-epidermal junction**

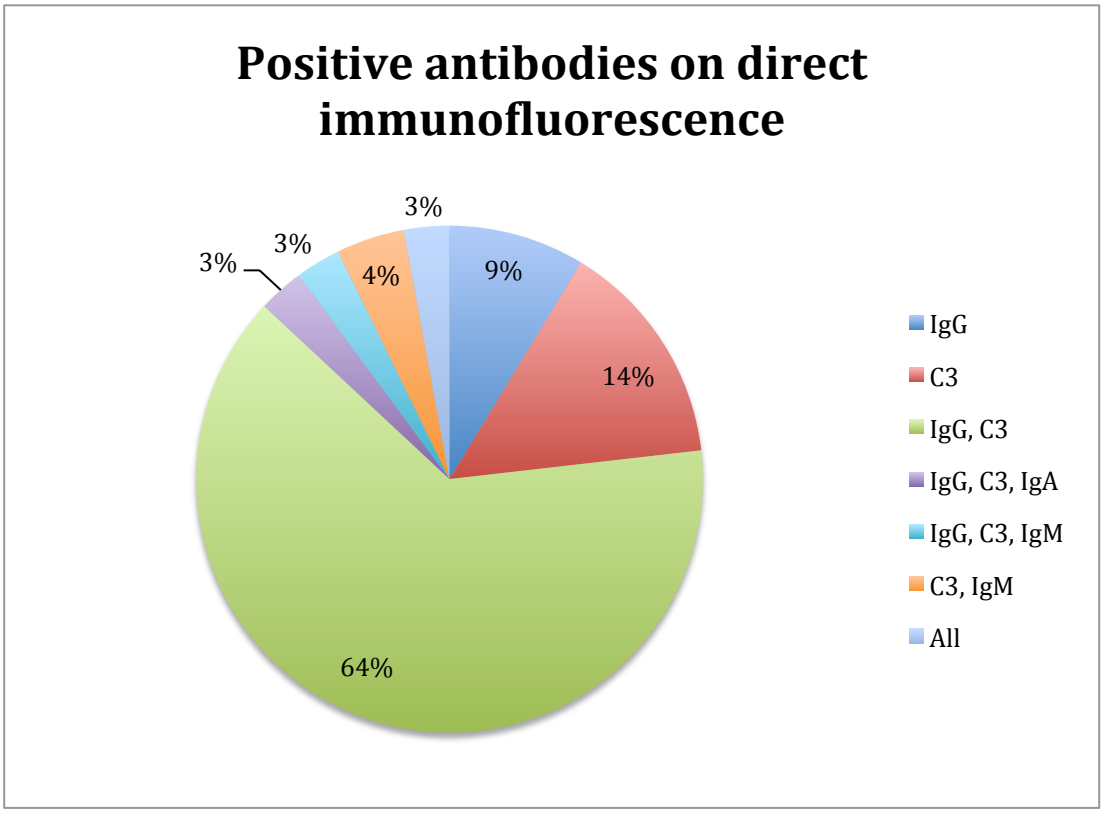
Positive antibodies on direct immunofluorescence
3%
3%
3%
4%
9%
14%
64%
IgG
C3
IgG, C3
IgG, C3, IgA
IgG, C3, IgM
C3, IgM
All

Figure 3

APPENDICES

1. Ethics approval

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6492 • **Facsimile** [021] 406 6411
Email: Sumayah.arieffdien@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

05 March 2015

HREC/REF: 120/2015

Prof N Khumalo
Division of Dermatology
G-23
NGSH

Dear Prof Khumalo

Project Title: THE USE OF COLLAGEN IV IMMUNOSTAINING IN THE DIAGNOSIS OF BULLOUS PEMPHIGOID (Mmed candidate- Roxanne Caron de Silva)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

It is a pleasure to inform you that the HREC has **formally approved** the above mentioned study.

Approval is granted for one year until the 28 March 2016.

Please submit a progress form, using the standardised Annual Report Form, if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.

We acknowledge that the following student-Dr Roxanne de Silva is also involved in this project.

Please note that the on-going ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the HREC REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.

Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

The Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

Hrec/ref:120/2015

UNIVERSITY OF CAPE TOWN
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FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

FHS017: Annual Progress Report / Renewal

Record Reviews/Audits/Collection of Biological Specimens/Repositories/Databases/Registries

HREC office use only (FWA00001637; IRB00001938)			
This serves as notification of annual approval, including any documentation described below.			
☑ Approved	Annual progress report	Approved until/next renewal date	28.3.2017
☐ Not approved	See attached comments		
Signature Chairperson of the HREC		Date Signed	9/3/2016

Principal Investigator to complete the following:

1. Protocol information

Date (when submitting this form)	08/03/2016		
HREC REF Number	120/2015	Current Ethics Approval was granted until	28/03/2016
Protocol title	The use of collagen IV immunostaining in the diagnosis of bullous pemphigoid		
Principal Investigator	Roxanne C. de Silva		
Department / Office Internal Mail Address	Dermatology, G23, Groote Schuur		
1.1 Does this protocol receive US Federal funding?		☐ Yes	☑ No

2. Protocol status (tick ✓)

☐	Research-related activities are ongoing
☑	Data collection is complete, data analysis only

Please indicate (in the block below) the titles and HREC reference numbers of any projects currently making use of the Database/registry/repository.

N/A

3. Protocol summary

Total number of records or specimens collected, reviewed or stored since the original approval		157
Total number of records or specimens collected, reviewed or stored since last progress report		N/A
Have any research-related outputs (e.g. publications, abstracts, conference presentations) resulted from this research? If yes, please list and attach with this report.	☐ Yes	☑ No

4. Signature

Signature of PI		Date	08/03/2016

2. British Journal of Dermatology author guidelines

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Online Submission
Patient Consent Form
CONSORT Checklist [req for RCTs]
STROBE Checklists (Cohort, Case-control, Cross-sectional) [req for observational studies]
Open Access Order Form
Peer-review Process Map
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1. ABOUT *BJD*

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The *British Journal of Dermatology* (*BJD*) strives to publish the highest quality dermatological research. In so doing, the journal aims to advance understanding, management and treatment of skin disease and improve patient outcomes.

BJD invites submissions under a broad scope of topics relevant to clinical and experimental research and publishes original articles, reviews, case reports and items of correspondence. The article categories within the journal are: cutaneous biology; clinical and laboratory investigations; contact dermatitis & allergy; dermatological surgery & lasers; dermatopathology; epidemiology & health services research; paediatric dermatology; photobiology; and therapeutics.

BJD is an official organ of the British Association of Dermatologists but attracts contributions from all countries in which sound research is carried out, and its circulation is equally international. The overriding criteria for publication are scientific merit, originality and interest to a multidisciplinary audience.

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The Journal aims to publish concise, high-quality review articles of recent advances in laboratory or clinical research. Review articles may be solicited by the Editor or may be submitted by authors for publication subject to peer review. Review articles must include an unstructured abstract (maximum 250 words), and should not exceed 3000 words of body text. Use of illustrations and figures is encouraged. Review articles must include bulleted statements (maximum 70 words) in answer to the following questions: what's already known about this topic?; what does this study add?

Original articles

Original articles are the Journal's primary mode of communication. Original articles must include a structured abstract (maximum 250 words), and should not exceed 3000 words of body text. Original articles must include bulleted statements (maximum 70 words) in answer to the following questions: what's already known about this topic?; what does this study add?

Manuscripts reporting randomised controlled trials (RCTs) must follow the **CONSORT** statement. RCTs will not be considered by *BJD* without submission of a completed **CONSORT checklist**. Manuscripts reporting observational studies must follow the **STROBE** guidelines. Observational studies will not be considered by *BJD* without submission of the relevant STROBE checklist.

Submissions reporting industry-sponsored clinical research are welcomed. The *British Journal of Dermatology* recommends all authors take account of the following guidelines when preparing their manuscript: *Ten Recommendations for Closing the Credibility Gap in Reporting Industry-Sponsored Clinical Research*.

For purposes of presentation only, accepted original articles are divided into the following sections:

Cutaneous biology
Clinical and laboratory investigations
Contact dermatitis and allergy
Dermatological surgery and lasers
Dermatopathology
Epidemiology and health services research
Genetics [1]
Paediatric dermatology
Photobiology
Therapeutics

Perspectives

The BJD welcomes submissions to the perspectives section covering a wide variety of topics relevant to contemporary dermatology healthcare and research. Such pieces can be opinion essays, which are similar in style to editorials, but are not tied to a particular article and clearly represent the views and opinions of the author(s) not the BJD. Perspectives articles can also address a range of social aspects of medicine and healthcare that are relevant to the practice of dermatology throughout the world. Perspectives should be concise, accessible, carefully crafted pieces; they are limited to 1,500-2000 words. Perspectives can include one figure or table and a maximum of 5-10 references. Perspectives are usually invited articles, but may also be submitted as unsolicited articles as long as they conform to the above instructions to authors.

Case reports

BJD includes only case reports of novel and extraordinary significance. Case reports must include an unstructured abstract and should not exceed 1200 words of body text with up to 15 references and 4 tables or figures. Case reports must include bulleted statements (maximum 70 words) in answer to the following questions: what's already known about this topic?; what does this study add?

Correspondence

The correspondence section (Letters to the Editor) includes various different types of letters. These include: BJD Research Letters; Rapid Response to Recently Published Original Articles (RRR-POA) published by the BJD; Opinion Pieces; Case Reports.
All items of correspondence should be formatted in one continuous section, with no bulleted statements or abstract. The BJD regards Research Letters as its' most prestigious form of correspondence. These should not exceed 1000 words, 15 references, two figures/tables. Rapid Response to Recently Published original articles should be scholarly, respectful to other authors, concise and to the point. They are intended to provide post-publication peer-review; as such, these letters are important and are published by fast track. Letters that are opinion pieces are also welcome; this type of letter provides

an opportunity to publish original ideas, innovations, scholarly opinions, debates and controversies at an early stage of academic development; such pieces must also be concise.
With the exception of Research Letters (see above), other forms of correspondence should not exceed 800 words, 10 references and two figures. Ideally, letters should be more concise than these limits. For all forms of correspondence, authors are advised to seek their own peer review by local scholars (who can be acknowledged) prior to submission. Additionally, all letters are subject to expert external peer review.

Cover picture (New)

The BJD is now seeking submissions for a new category of publication, the "cover picture" to be printed on the front cover of the journal. Photographs or images should be visually arresting, of high quality and of educational value. The image should be submitted with a concise, scholarly caption (of up to 100 words) explaining the image, which will be printed on the inside of the front cover. Examples of potential cover pictures include clinical photographs of normal or diseased skin, images of a diagnostic technique (for example, histopathological images, immunostains, electron microscopy) or device or a historical image. Cover pictures will be selected for their visual impact, scientific merit, originality and relevance to the journal readership.

Editorials/Commentaries

Editorials and commentaries are typically commissioned by the Editors. However, suggestions for such articles are welcomed and should be directed to the **Editorial Office**.

3. SUBMISSION OF MANUSCRIPTS

All submissions should be made online at the *BJD* **ScholarOne Manuscripts** site (formerly known as Manuscript Central). New users should first create an account. Once a user is logged onto the site, submissions should be made via the Author Centre.

Submissions **must** be accompanied by a completed **Author Consent Form**. Completed forms **must** be uploaded to ScholarOne Manuscripts at the same time as manuscript submission using file designation 'Supplementary files not for review'.

4. PREPARATION OF MANUSCRIPTS

Manuscripts must be written in British English.
Manuscript text must be saved in Word (.doc or .docx) or Rich Text Format (.rtf). Do not submit text in PDF format (.pdf). Figures must be saved as separate figure files. GIF, JPEG, PICT or Bitmap files are acceptable for submission, but only TIFF or EPS files are suitable for printing. After acceptance, you will be contacted to provide print-quality figures if you have not already done so. NOTE: If you're able to supply figures PDF format (.pdf)

only they must be distilled using the 'Print Optimised' option.
Abbreviations must be defined when first used in the abstract and in the main text, as well as when first used in table and figure captions.
Manuscripts must be as succinct as possible. Repetition of information or data in different sections of the manuscript must be carefully avoided. Text must comply with the word limits defined in Section 2, and, where appropriate, include:

Title Page
The first page of all manuscripts should contain the following information:

1) the title of the paper
2) a running head not exceeding 70 characters (not needed for correspondence items)
3) manuscript word, table and figure count
4) names of authors as initial(s) followed by surnames
5) names of the institutions at which the research was conducted, clearly linked to respective authors using superscript Arabic numbers
6) name, address, telephone and fax number, and email address of corresponding author
7) a statement of all funding sources that supported the work
8) any conflict of interest disclosures (see Section 5)
9) bulleted statements (maximum 70 words) in answer to each of the following questions: what's already known about this topic?; what does this study add? (Not applicable to Correspondence items.)

Abstracts
Authors submitting original articles should note that structured abstracts are required. The structured abstract should adopt the format: Background, Objectives, Methods, Results, Conclusions.
Review articles and case reports require abstracts but they need not be structured.
Abstracts should contain no citations to previously published work.
Correspondence and gene corner articles do not require abstracts.

Text
This should in general, but not necessarily, be divided into sections with the headings: Summary, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, References, Figure legends.

Tables and Figures
Tables should not be inserted in the appropriate place in the text but should be included at the end of the manuscript, each on a separate page.
Figures must be submitted as a separate file or files.
Tables and figures should be referred to in text as follows: Fig. 1, Figs 2–4; Table 1, Table 2. Each table and/or figure must have a legend that explains its purpose without reference to the text. Where a figure has more than one panel, each panel should be labelled in the top left-hand corner using lower case letters in parentheses, i.e. ‘(a)’, ‘(b)’ etc. and a brief description of each panel given in the figure legend.

Colour illustrations are welcomed and all colour is published free of charge to the author.
Authors are themselves responsible for obtaining permission to reproduce previously published figures or tables. When an individual is identifiable in a photograph written permission must be obtained (see Section 5 below).

Electronic Artwork
Vector graphics (e.g. line artwork) should be saved in Encapsulated Postscript Format (EPS), and bitmap files (e.g. photographs) in Tagged Image File Format (TIFF). Line art must be scanned at a minimum of 800 dpi, photographs at a minimum of 300 dpi.

References
References should be in Vancouver format and appear as consecutive, unbracketed superscript numbers in the text, e.g. 'in our previous reports[1,2] and those of Smith *et al*. [3–5]' and should be listed numerically in the reference list at the end of the article.
Format references as below, using standard (Medline) abbreviations for journal titles. If more than four authors, include the first three authors followed by et al.

1 de Berker DAR, Baran R, Dawber RPR. The nail in dermatological diseases. In: *Baran and Dawber's Diseases of the Nails and their Management*(Baran R, Dawber RPR, de Berker DAR, Haneke E, Tosti A, eds), 3rd edn. Oxford: Blackwell Science Ltd, 2001; 172–92.

2 Shuster S. The nature and consequence of Karl Marx's skin disease. *Br J Dermatol* 2008; **158**:1–3.

3 Graham-Brown R, Burns T. *Lecture Notes: Dermatology*. Oxford: Wiley-Blackwell, 2006.

4 Smith A. (1999) Select committee report into social care in the community [WWW document]. URL http://www.dhss.gov.uk/reports/report015285.html [accessed on 7 November 2003].

We recommend the use of a tool such as EndNote for reference management and formatting. EndNote reference styles can be searched for here: **http://www.endnote.com/support/enstyles.asp**.

Reporting Standards
Manuscripts reporting randomised controlled trials (RCTs) must follow the **CONSORT** statement. RCTs will not be considered by *BJD* without submission of a completed **CONSORT checklist**. Checklists should be uploaded during manuscript submission using file designation 'Supplementary files for review'.
Manuscripts reporting observational studies must follow **STROBE** guidelines. Observational studies will not be considered by *BJD* without submission of a completed STROBE checklist (**cohort studies**, **case-control studies**, **cross-sectional studies**). Checklists should be uploaded during manuscript

submission using file designation 'Supplementary files for review'.

Supporting Information
BJD encourages the submission of underlying datasets, appendices, movie files, etc. as online-only supporting information. Supporting information should be uploaded during manuscript subission (see Section 3) using file designation 'Supplementary files for review'.
Supporting information should be important ancillary information that is relevant to the parent article but which does not or cannot appear in the printed edition of the Journal. Supporting information will be published as submitted, and will not be corrected or checked for scienfitic content, typographical errors or functionality.

5. DECLARATIONS

Original Publication

Submission of a manuscript will be held to imply that it contains original unpublished work and is not being submitted for publication elsewhere at the same time. The author must supply a full statement to the Editor about all submissions and previous reports that might be regarded as redundant or duplicate publication of the same or very similar work. *BJD* employs a plagiarism detection system. By submitting your manuscript, you accept that your manuscript may be screened for plagiarism against previously published works.

Conflicts of Interest

Authors are responsible for disclosing all financial and personal relationships between themselves and others that might be perceived by others as biasing their work. To prevent ambiguity, authors must state explicitly whether potential conflicts do or do not exist.

Ethics

When reporting experiments on human subjects, indicate whether the procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional or regional) and with the Helsinki Declaration of 1975, as revised in 1983. Do not use patients' names, initials or hospital numbers, especially in illustrative material. When reporting experiments on animals, indicate whether the institution's or a national research council's guide for, or any national law on, the care and use of laboratory animals was followed. A statement describing explicitly the ethical background to the studies being reported should be included in all manuscripts in the Materials and Methods section. Ethics committee or institutional review board approval should be stated.
Patients have a right to privacy that should not be infringed without informed consent. Identifying information should not be published in written descriptions, photographs and pedigrees unless the information is essential for scientific purposes and the patient (or parent or guardian) gives written

informed consent for publication. Identifying details should be omitted if they are not essential but patient data should never be altered or falsified in an attempt to attain anonymity. Complete anonymity is difficult to achieve and informed consent should be obtained if there is any doubt. For example, masking the eye region in photographs of patients is inadequate protection of anonymity.

Authorship

All persons designated as authors should qualify for authorship and all those who qualify should be listed. Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content. One or more authors should take responsibility for the integrity of the work as a whole, from inception to published article. Authorship credit should be based only on 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; 3) final approval of the version to be published. Conditions 1, 2 and 3 must all be met. Acquisition of funding, the collection of data or general supervision of the research group, by themselves, do not justify authorship. All others who contributed to the work who are not authors should be named in the Acknowledgments section.

Committee on Publication Ethics (COPE)

As a member of the Committee on Publication Ethics (COPE), adherence to these submission criteria is considered essential for publication in the *BJD*; mandatory fields are included in the online submission process to ensure this. If, at a later stage in the submission process or even after publication, a manuscript or authors are found to have disregarded these criteria, it is the duty of the Editor to report this to COPE. COPE may recommend that action be taken, including but not exclusive to, informing the authors' professional regulatory body and/or institution of such a dereliction.
The website for COPE may be accessed at: **http://publicationethics.org**